THE EFFECT OF CERTAIN METABOLITES ON THE GROWTH OF INFLUENZA VIRUS IN THE DEVELOPING HEN'S EGG

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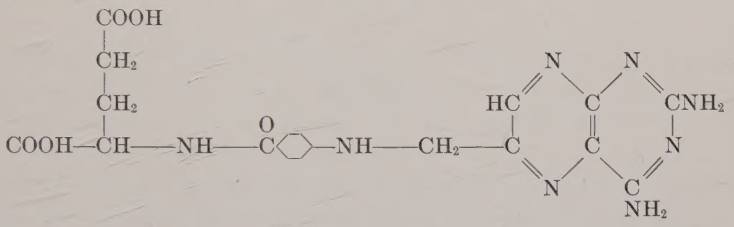
Since pteroylglutamic acid (folic acid) has been proved to have an essential role in the growth of bacteria, protozoa, insects, birds and mammals (Jukes, 1948), it was considered desirable to determine whether it is required in the metabolism of the influenza virus. In order to test this possibility it was necessary to tie up the folic acid in the medium in which the influenza virus was growing. Several antagonists to folic acid have recently been discovered and it therefore seemed feasible to tie up the folic acid present in a developing egg by the use of these compounds and then determine whether the virus would grow in such an enzyme-deficient system. 4-aminopteroylglutamic acid (aminopterin) was discovered in 1947 and has an NH_2 group on the 4 position of the pteridyl nucleus instead of the OH group of folic acid (Seeger, 1947).



Structural Formula for Aminopterin

Aminopterin is a true folic acid antagonist capable of producing an acute and absolute folic acid deficiency even in the presence of folic acid (Philips, 1949). It has been shown by this worker that the use of an excess of added folic acid over and above that originally present does not alleviate the effect produced by this substance. A dose of aminopterin thus had to be determined which would largely deplete the egg of its enzymes requiring folic acid but which would not kill the egg during the period required for the virus and its growth within the egg.

Karnofsky and his associates (1949) noted some effects which aminopterin produced in the chick embryo. These workers determined an LD_{50} dose representing an amount of aminopterin which caused characteristic abnormalities but which allowed half the eggs injected to survive beyond 9 days of age. The amount which conformed to these specifications was 0.003 milligram per egg. They found that this dosage produced conspicuous abnormalities which were most striking in those eggs surviving eight to ten days after the injection.

In order to determine whether folic acid was necessary for the growth of the influenza virus, certain modifications of Karnofsky's conditions had to be made in order to meet the generally accepted requirements for the growth of the virus. It has been shown that 10-day-old embryonating eggs are most suitable for the development of the virus and a two-day period of incubation following inoculation of the virus gives a maximum yield of virus in the allantoic fluid.

Materials and Methods

Five-day-old embryonating eggs were selected to receive the aminopterin dose since according to our experience eggs of this age survived the trauma produced by the injection much better than the 4-day-old eggs used by Karnofsky (1949). Preliminary experimentation demonstrated that 0.0025 milligram of aminopterin was consistently sufficient to produce the abnormalities described by Karnofsky (1949). Since this was smaller than their LD_{50} dose of 0.003 milligram, it could be expected to favor the survival of eggs through the desired 7-day experimental period.

Sterile 1 milligram vials of aminopterin were generously furnished by the Lederle Laboratories. The contents of one vial were dissolved in 40 ml. of sterile distilled water and 0.1 ml. of this solution containing 0.0025 milligram of aminopterin was injected into each egg. The method of injection was essentially the same as that used by Karnofsky (1949). Control eggs received distilled water instead of the aminopterin solution. After injection with aminopterin or distilled water the eggs were incubated for another five days. On the tenth day the live eggs were inoculated with type B influenza virus (LEE strain) and incubated at 35.5°C–36.5°C for about 48 hours. The allantoic fluid was then harvested and the titre of the virus was determined on each individual fluid by the chicken red cell agglutination method of Salk (1944).

Results

Of a total of 193 five-day-old eggs injected with aminopterin, 51 (26%) died before the tenth day. A total of 82 eggs of the same age were injected with distilled water. Twelve (14%) of these died before the tenth day. This indicates that some eggs in the aminopterin group died from trauma rather than from the effects of aminopterin alone.

The majority of the embryos which received aminopterin showed conspicuous stunting, increasing in proportion to the period of survival. In line with the findings of Karnofsy *et al.* (1949), the deformities usually present were a flattened head, smaller-than-normal eyes, seriously de-

formed mouth parts especially the mandible, exteriorization of the viscera, stunted and incompletely formed wings and feet, and various degrees of generalized hemorrhage and edema.

Table I compares the growth of influenza virus B (LEE strain) in aminopterin-treated eggs with that in control eggs as measured by the hemagglutinating titre after 48 hours of incubation. A titre greater than 1/160 indicated a fairly good growth of the virus while a titre of between 1/20 and 1/160 indicated scanty growth of virus. A titre of less than 1/20 was accepted as evidence of very poor growth or no growth at

TABLE I
GROWTH OF LEE INFLUENZA VIRUS IN AMINOPTERIN-TREATED AND CONTROL EGGS AS MEASURED BY CCA TITRES AFTER 48 HOURS INCUBATION

No. of Eggs	Treatment	CCA Titres 1/20	1/20–1/80	1/160 and Higher
142	Inoculated with aminopterin 5 days before virus inoculation	66 (46%)	17 (12%)	59 (41%)
70	Control group inoculated with dist. H_2O 5 days before virus inoculation	26 (37%)	8 (11%)	36 (51%)

all. It is apparent that there is no conspicuous difference in the agglutinating titres of the aminopterin-treated and control eggs.

These results therefore seem to indicate that folic acid plays no role in the development of the influenza virus within the embryonating egg.

DISCUSSION AND CONCLUSIONS

According to Karnofsky and his co-workers (1949) the abnormalities produced by aminopterin result from a folic acid deficiency. These same abnormalities were produced in this study by a slightly smaller dose in order to fit the experimental conditions. If the enzyme system requiring folic acid was the pathway involved in the growth of the influenza virus, then the blocking of this pathway would presumably lead to a partial or complete cessation of virus production. This appears not to be the case, for as long as the egg remains alive the propagation of virus is not appreciably hindered.

Individual eggs, however, seem to vary in their ability to withstand the effects of aminopterin. Eggs dying during an eight-day period following injection of aminopterin show much greater changes than those surviving this period. It is reasonable to believe that this variation is

related to the amount of folic acid which is laid down in the egg during its formation and which is consequently not completely bound by the amount of aminopterin injected.

The embryonating egg is a complex of various enzyme systems and if it finds one system blocked or deficient, it may be able to compensate, within limits, by increasing its usage of other systems. This would be essentially a defense mechanism and would be useful in prolonging the life of the embryo only up to the point where the lacking or deficient enzyme system is necessary, or required in larger amounts. Then if this system is not functioning adequately enough, the embryo will die. Thompson (1949) has shown that aminopterin fails to show more than minimal inhibition in relation to the production of vaccinia virus. His is essentially the same result as that here obtained with the influenza virus. The experiments of Thompson, however, were performed in vitro (Maitland roller tube tissue culture). The experiments with the influenza virus herein reported were performed in vivo, that is, in the embryonating egg. However, the most likely conclusion is the same, namely, that the multiplication of influenza virus utilizes a pathway which is not blocked by the folic acid antagonist aminopterin.

VITA

The writer was born in Webster City, Iowa, of British parents. He attended grammar school and part of high school in England. Returned to the United States in 1923 and finished high school in Webster City. Attended the State University of Iowa and received the B.A. degree in 1932. Received the M.Sc. degree from Iowa State College in 1937. Returned to England in 1937 for one year and while there attended the London School of Hygiene and Tropical Medicine. Came back to the United States in 1938 and attended the University of Illinois in Urbana. Obtained an appointment as Bacteriologist in The Illinois State Health Dept., Chicago, in 1940. Joined the U. S. Army — Sn.C., in 1942. Served 3 years overseas in the North African and Italian Theatres in charge of the Biochemistry Section of the 2nd Medical Laboratory.

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